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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/668,073	09/19/2003	Andrew H. Segal	11111/2003F	3004
29933	7590	03/11/2008	EXAMINER	
PALMER & DODGE, LLP			BLUMEL, BENJAMIN P	
KATHLEEN M. WILLIAMS				
111 HUNTINGTON AVENUE			ART UNIT	PAPER NUMBER
BOSTON, MA 02199			1648	
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			03/11/2008	PAPER

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

<b>Office Action Summary</b>	<b>Application No.</b>	<b>Applicant(s)</b>	
	10/668,073	SEGAL ET AL.	
	<b>Examiner</b>	<b>Art Unit</b>	
	BENJAMIN P. BLUMEL	1648	

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*  
**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) Responsive to communication(s) filed on 12 December 2007.
- 2a) This action is **FINAL**.                            2b) This action is non-final.
- 3) Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

- 4) Claim(s) 1-82 is/are pending in the application.
- 4a) Of the above claim(s) 5-9, 13, 14 and 40-78 is/are withdrawn from consideration.
- 5) Claim(s) \_\_\_\_\_ is/are allowed.
- 6) Claim(s) 1-4, 10-12, 15-39 and 79-82 is/are rejected.
- 7) Claim(s) \_\_\_\_\_ is/are objected to.
- 8) Claim(s) \_\_\_\_\_ are subject to restriction and/or election requirement.

**Application Papers**

- 9) The specification is objected to by the Examiner.
- 10) The drawing(s) filed on \_\_\_\_\_ is/are: a) accepted or b) objected to by the Examiner.  
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

**Priority under 35 U.S.C. § 119**

- 12) Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).  
 a) All    b) Some \* c) None of:
  1. Certified copies of the priority documents have been received.
  2. Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
  3. Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- 1) Notice of References Cited (PTO-892)
- 2) Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) Information Disclosure Statement(s) (PTO/SB/08)  
 Paper No(s)/Mail Date \_\_\_\_\_
- 4) Interview Summary (PTO-413)  
 Paper No(s)/Mail Date \_\_\_\_\_
- 5) Notice of Informal Patent Application
- 6) Other: \_\_\_\_\_

## **DETAILED ACTION**

Applicants are informed that the rejections of the previous Office action not stated below have been withdrawn from consideration in view of the Applicant's arguments and/or amendments.

### ***Election/Restrictions***

Claims 5-9, 13, 14 and 40-78 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to nonelected species, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on December 21, 2006.

Claims 1-4, 10-12, 15-39 and 79-82 are examined on the merits.

### ***Response to Arguments***

Applicant's arguments with respect to claims 1-4, 10-12, 15-39 and 79-82 have been considered but are moot in view of the new ground(s) of rejection. However, some of the previously cited references are still applied below with corresponding responses by the Examiner.

### ***Double Patenting***

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

**(New Rejection)** Claims 1-3, 10-12, 30-34, 36, 37 and 39 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-7 and 10-16 of copending Application No. 10/667,193 in view of Hoo (US 5,891,432).

The co-pending invention is drawn to a method of modulating an immune response in a mammal/human by administering a composition containing an antigen bearing target (cell or virus) and a multi-functional molecule containing a first amino acid sequence of a cell-surface binding moiety and a second amino acid sequence comprising a ligand for a cytokine receptor (GM-CSF). The multi-functional molecule is a fusion polypeptide wherein the first amino acid sequence is N-terminal or C-terminal to the second amino acid sequence. The ligand is specific for a mouse cell surface polypeptide, such as leukocytes, professional antigen presenting cells, such as dendritic cells. The multi-functional molecule can be bound or unbound to the antigen bearing target. Therefore, in view of Hoo who teaches the generation of a multi-functional molecule with a naturally occurring lectin fused with murine GM-CSF, the co-pending invention is an obvious variant of the instant one.

This is a provisional obviousness-type double patenting rejection.

***Claim Rejections - 35 USC § 102***

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

**(New Rejection)** Claims 1-3, 10-12, 17, 27, 28, 30-36, 81 and 82 are rejected under 35 U.S.C. 102(b) as being anticipated by Hoo (US 5,891,432) as evidenced by Erbe et al. (Journal of Cell Biology, 1993) and Cantrell et al. (PNAS, 1985).

The claimed invention is drawn to a method of modulating an immune response in a mammal/human by administering a composition containing an antigen bearing target and a multi-functional molecule containing a first amino acid sequence of a naturally occurring lectin and a second amino acid sequence comprising a ligand for a cytokine receptor. The multi-functional molecule is a fusion polypeptide wherein the first amino acid sequence is N-terminal or C-terminal to the second amino acid sequence. The ligand is specific for a mouse cell surface polypeptide, such as leukocytes, professional antigen presenting cells, such as dendritic cells. The multi-functional molecule can be bound or unbound to the antigen bearing target. It is noted that on page 2 of the specification, "an "antigen bearing target" is an entity which comprises an antigen. As used herein an "antigen bearing target" includes, for example, a whole cell which expresses an antigen a cell fraction comprising an antigen, a membrane fraction comprising an antigen, a virus comprising an antigen, a viral particle comprising an antigen, or an antigen, e.g. a polypeptide antigen, which may be free of any other cell-derived or virus-derived material. Cellular fractions may be prepared using methods known to those of skill in the

art such as those taught in Cell Biology A Laboratory Handbook (Academic Press 1994 Editor J. E. Celis ISBN 0-12-164715-3)."

Hoo teaches the use of antigen bearing targets, such as cells that express a fusion polypeptide based on one amino acid sequence of P-selectin fused with either a second amino acid sequence of murine GM-CSF or IL-2. The P-selectin employed (a natural lectin) inherently has a carbohydrate binding domain as evidenced by the teachings of Erbe et al. In addition, the murine GM-CSF used contains at least five contiguous amino acids which the human form also contains as evidenced by Cantrell et al. and that professional APCs, such as dendritic cells, contain receptors for GM-CSF. Hoo also teaches that the fusion polypeptides can be formed by fusing the lectin to the N- or C- terminus of the GM-CSF peptide and that the fusion polypeptide can be either attached or unattached to the antigen bearing target (i.e., cell). Therefore, Hoo anticipate the claimed invention as evidenced by Erbe et al. and Cantrell et al.

***Claim Rejections - 35 USC § 103***

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

**(New Rejection)** Claims 1-4, 10-12, 15, 16, 18-39, 81 and 82 are rejected under 35 U.S.C. 103(a) as being unpatentable over Hoo *supra*, Erbe et al.*supra*, Cantrell et al. *supra*, Faulkner et al. (International Immunology, 2001), Operschall et al. (Journal of Clinical Virology, 1999) and Nobusawa et al. (Virology, 1991).

The claimed invention is drawn to a method of modulating an immune response in an animal with a 10 amino acid fragment of influenza hemagglutinin (HA) fused with a ligand for a cytokine receptor. The animal could be a mammal, such as a human.

Additionally, the HA fragment is of the influenza virus A/PR/8/34 and is N-terminal to the ligand. The HA, a naturally occurring Lectin, is capable of binding to a carbohydrate structure with sialic acids. The claimed invention also involves an influenza virus hemagglutinin H2 or H3 or a HA of an influenza that does not infect humans. In addition, the ligand is mouse or human GM-CSF and is fused with the HA antigen at the N- or C-terminus of GM-CSF. It is noted that on page 2 of the specification, "an "antigen bearing target" is an entity which comprises an antigen. As used herein an "antigen bearing target" includes, for example, a whole cell which expresses an antigen a cell fraction comprising an antigen, a membrane fraction comprising an antigen, a virus comprising an antigen, a viral particle comprising an antigen, or an antigen, e.g. a polypeptide antigen, which may be free of any other cell-derived or virus-derived material. Cellular fractions may be prepared using methods known to those of skill in the art such as those taught in Cell Biology A Laboratory Handbook (Academic Press 1994 Editor J. E. Celis ISBN 0-12-164715-3)."

The teachings of Hoo are summarized above, however Hoo does not discuss the use of influenza hemagglutinin as the lectin.

The teachings of Erbe et al. are discussed above.

The teachings of Cantrell et al. are discussed above.

Faulkner et al. teach the development of a chimeric vaccine comprising 10 amino acid region of HA from Influenza virus A/PR/8/34 (PR8) linked to IL-2 and the importance of researching other chimeric cytokine-antigen vaccines that provide the therapeutic effects of the cytokine with the antigenic properties of the antigen in addition to improving the half-life of the cytokine *in vivo*. Some examples of cytokine candidates

are IFN- $\gamma$ , GM-CSF, IL-4, and IL-10 since the respective receptors are expressed by Dendritic Cells (DCs), which also function as antigen presenting cells, as also discussed by Faulkner et al. Faulkner et al. further teach the use of the HA-IL-2 chimeric in the activation of bone marrow-derived dendritic cells with compared to treatments with separated HA and IL-2. Even though did not administer the chimeric vaccine to an animal, Faulkner et al. observed an increased T cell activation by way of antigen presentation of the chimeric composition from DCs and they also disclose that previous studies pertaining to *in vivo* activity of similar chimeras have been analyzed.

Operenschall et al. teach the co-administration of plasmid DNA that encodes Influenza A/PR/8/34 hemagglutinin and mouse GM-CSF to mice in order to protect against viral infection. Operenschall et al. observed that the cytokine-antigen combination possess adjuvant properties.

Nobusawa et al. teach the comparison of 13 HA serotypes of Influenza A viruses. In particular, Nobusawa et al. H2, H3, H8 and H12, of which, H8 and H12 serotype viruses are not known to have infected humans as of yet.

It would have been obvious to one of ordinary skill in the art to modify the methods taught by Hoo (and evidenced by Erbe et al. and Cantrell et al.) and Faulkner et al. in order to link hemagglutinin from PR8 to GM-CSF as part of immunogenic composition also containing an antigen bearing target as discussed above. One would have been motivated to do so, given the suggestion by Hoo and Faulkner et al. that the method be used to produce fusion polypeptides with lectins fused to cytokines, particularly using influenza HA and GM-CSF. There would have been a reasonable expectation of success, given the knowledge that the co-administration of influenza HA

and mouse GM-CSF have adjuvant related properties, as taught by Operschall et al., and also given the knowledge that various Influenza A hemagglutinin antigens (H2, H3, H8 and H12) are known based on sequence analysis, as taught by Nobusawa et al. Thus the invention as a whole was clearly *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

**Responses:**

Applicants argue that Faulkner et al. do not teach the claimed antigen bearing target in conjunction with the multi-functional molecule of a carbohydrate binding HA fused to GM-CSF. Applicants also argue that Faulkner et al. do not teach a vaccine comprising the above mentioned composition being administered. In response, it is acknowledged that Faulkner et al. do not teach the exactly claimed composition. With regard to the statement pertaining to the lack of Faulkner et al. to teach a vaccine comprising the same, such a limitation is not claimed at this point, therefore Faulkner et al. do not need to teach a vaccine. Furthermore, Faulkner et al. do teach how to make a fusion polypeptide containing an influenza PR8 HA segment linked to IL-2 which can be used to activate dendritic cells.

Applicants further argue that the teachings of Faulkner et al. do not suggest to substitute their HA fragment with a sialic acid binding domain of HA and if they did, one would expect the binding affinity to inhibit the interaction between the GM-CSF and its cellular receptor. However, even though Hoo employ a different lectin than Faulkner et al. he is able to effectively use the multi-functional fusion polypeptide without inhibiting the GM-CSF activity.

Applicants argue that Operschall et al. do not teach the fusion construct of influenza HA and GM-CSF, only dual plasmid immunization. In response, it is acknowledged that Operschall et al. do not teach the claimed fusion polypeptide, but they do teach the adjuvant effects that expressed GM-CSF and influenza HA have on protecting mice from influenza infections.

**(New Rejection)** Claims 1-3, 10, 79 and 80 are rejected under 35 U.S.C. 103(a) as being unpatentable over Hoo *supra*, Erbe et al. *supra*, Cantrell et al. *supra*, Guillett et al. (European Journal of Biochemistry, 2002), Robinson et al. (Proceedings of the National Academy of Science, 1998).

The claimed invention is drawn to a method of modulating an immune response in a mammal/human by administering a composition containing an antigen bearing target and a multi-functional molecule containing a first amino acid sequence of a naturally occurring lectin and a second amino acid sequence comprising a ligand for a cytokine receptor. The multi-functional molecule is a fusion polypeptide wherein the first amino acid sequence is N-terminal or C-terminal to the second amino acid sequence and are linked through a Glycine-Serine linker. The ligand is specific for a mouse cell surface polypeptide, such as leukocytes, professional antigen presenting cells, such as dendritic cells. The multi-functional molecule can be bound or unbound to the antigen bearing target. It is noted that on page 2 of the specification, "an "antigen bearing target" is an entity which comprises an antigen. As used herein an "antigen bearing target" includes, for example, a whole cell which expresses an antigen a cell fraction comprising an antigen, a membrane fraction comprising an antigen, a virus comprising an antigen, a

viral particle comprising an antigen, or an antigen, e.g. a polypeptide antigen, which may be free of any other cell-derived or virus-derived material. Cellular fractions may be prepared using methods known to those of skill in the art such as those taught in Cell Biology A Laboratory Handbook (Academic Press 1994 Editor J. E. Celis ISBN 0-12-164715-3)."

The teachings of Hoo are summarized above, however he does not teach the claimed Glycine-Serine linker of claims 79 and 80.

The teachings of Erbe et al. are discussed above.

The teachings of Cantrell et al. are discussed above.

Guillet et al. teach linking of a cytokine, cardiotrophin-like cytokine (CLC), with a recombinant neurotrophic factor (CNTF) receptor via a Glycine-Serine linker ( $G_4S_2$ ). Guillet et al. observed an increase in stability among the chimeric complex.

Robinson et al. teach the identification of an ideal Glycine-Serine linker length and composition for the Arc repressor dimer. Robinson et al. discuss that identifying a linker, which improves desired properties (i.e. flexibility, stability, increased *in vivo* half-life) of a protein complex would prove to be a very important discovery. Through their random Arc-linker-Arc constructs, Robinson et al. identified an ideal linker with 7 serines and 9 glycines.

It would have been obvious to one of ordinary skill in the art to modify the methods taught by Hoo (and evidenced by Erbe et al. and Cantrell et al.) in order to use a Gly-Ser based linker in forming the fusion polypeptide. One would have been motivated to do so, given the suggestion by Hoo that the method be used to produce a fusion protein comprising a cell membrane binding protein to a cytokine through linkage domain.

There would have been a reasonable expectation of success, given the knowledge that the stability of a cytokine-heterologous protein chimera improved by a 10-mer linker of Glycine-Serine and in the case of a recombinant repressor which is stabilized by a 16-mer Glycine-Serine linker, as taught by Guillett et al. and Robinson et al., respectively. Thus the invention as a whole was clearly *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

***Summary***

No claims are allowed.

***Conclusion***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to BENJAMIN P. BLUMEL whose telephone number is (571)272-4960. The examiner can normally be reached on M-F, 8-4:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Bruce Campell can be reached on 571-272-1600. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/BENJAMIN P BLUMEL/  
Examiner  
Art Unit 1648

/Bruce Campell/  
Supervisory Patent Examiner, Art Unit 1648

<b>Notice of References Cited</b>		Application/Control No.	Applicant(s)/Patent Under Reexamination	
		10/668,073	SEGAL ET AL.	
Examiner		Art Unit		Page 1 of 1
BENJAMIN P. BLUMEL		1648		

**U.S. PATENT DOCUMENTS**

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,891,432	04-1999	Hoo, William Soo	424/93.21
*	B	US-2004/0170960	09-2004	Segal et al.	435/005
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

**FOREIGN PATENT DOCUMENTS**

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

**NON-PATENT DOCUMENTS**

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Erbe et al., P- and E-Selectin Use Common Sites for Carbohydrate Ligand Recognition and Cell Adhesion, 1993, The Journal of Cell Biology, Vol. 120, No. 5, pages 1227-1235.
	V	Cantrell et al., Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor (bone marrow colony assay/cDNA cloning/yeast expression), 1985, PNAS, Vol. 82, pages 6250-6254.
	W	
	X	

\*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)  
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

## Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor

(bone marrow colony assay/cDNA cloning/yeast expression)

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Communicated by Lewis H. Sarett, May 15, 1985

**ABSTRACT** Human granulocyte/macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that is essential for the *in vitro* proliferation and differentiation of precursor cells into mature granulocytes and macrophages. In this report we have used a mouse GM-CSF cDNA clone to isolate human GM-CSF clones from libraries made from HUT-102 messenger RNA and mitogen-stimulated T-lymphocyte messenger RNA. The human cDNA clones contained a single open-reading frame encoding a protein of 144 amino acids with a predicted molecular mass of 16,293 daltons and showed 69% nucleotide homology and 54% amino acid homology to mouse GM-CSF. One of these cDNA clones was shown to direct the synthesis of biologically active GM-CSF using a yeast expression system. The gene for human GM-CSF appears to exist as a single-copy gene.

The growth and differentiation of hematopoietic cells is mediated by a number of glycoproteins, collectively known as colony-stimulating factors (CSFs), including granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), and multi-CSF (interleukin 3) (1, 2). There has been considerable difficulty in defining these regulatory proteins, partly because they have been obtained from a variety of sources and characterized by their capacity to generate hematopoietic cells of various lineages *in vitro*. An understanding of the biology and biochemistry of these CSFs is further complicated by their existence in small quantities. Molecular cloning and expression of these factors should lead to a better definition of their structure and biological activities and exploration of their possible therapeutic uses.

GM-CSF is a glycoprotein that is required for the production of granulocytes and macrophages from normal bone marrow and appears to regulate the activity of mature, differentiated granulocytes and macrophages (1, 3, 4). Human GM-CSF, which has been isolated recently from the Mo T-lymphoblast cell line, has been shown to modulate the activities of mature neutrophilic granulocytes and appears to be identical to a neutrophil migration-inhibition factor from T lymphocytes (NIF-T) (5). A murine GM-CSF cDNA clone has been obtained following the determination of a partial amino-terminal sequence for the protein purified from mouse lung-conditioned medium (6, 7).

We report here the molecular cloning of human GM-CSF from cDNA libraries prepared from the HUT-102 cell line and from mitogen-stimulated T lymphocytes. The human sequences were isolated by hybridization with a mouse GM-CSF cDNA probe and were shown to direct the synthesis of biologically active GM-CSF using a yeast expression system.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

## MATERIALS AND METHODS

**Construction and Analysis of Libraries.** Polyadenylated messenger RNA was isolated from human peripheral blood T lymphocytes stimulated with concanavalin A (Con A) and phorbol myristate acetate (PMA) at 20 µg/ml and 10 ng/ml, respectively, and from the mouse T-lymphoma cell line LBRM-33-5A4 (ATCC-CRL-8080) stimulated with 1% phytohemagglutinin M (PHA-M). Procedures for RNA purification and cDNA library construction have been described (8).

Small-scale plasmid DNA preparations from pools representing approximately  $1 \times 10^5$  transformants were digested with *Pst* I, electrophoresed on 0.8% agarose gels, blotted onto nitrocellulose filters, and hybridized either with cDNA probes ( $^{32}$ P-labeled by nick-translation) or with oligonucleotide probes (labeled with T4 polynucleotide kinase). Hybridizations were for 16 hr at 55°C in 6× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 0.1% sarcosyl, 5× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 0.5% Nonidet P-40, 100 µg of denatured salmon sperm DNA per ml, and probe at  $10^6$  cpm/ml. Filters were washed extensively in 6× NaCl/Cit at room temperature, and then washed for 1 hr at 42°C and for 1.5 hr at 55°C before autoradiography. Positive pools containing the largest hybridizing cDNAs were subdivided, and the process was repeated until pools of 2,000–10,000 transformants were obtained. Then these pools were used in colony filter hybridization experiments (9) to identify transformants that hybridized strongly with the probes.

DNA from the cDNA clones was subcloned into M13 mp18 and mp19 (10) and sequenced by the chain-termination method (11) as described (12, 13).

**Expression of Human GM-CSF in Yeast.** An expression vector (Fig. 1) was constructed which included pBR322 sequences, the *TRP1* gene of yeast for tryptophan selection, the yeast 2-µm origin of replication, and the yeast  $\alpha$ -factor promoter and leader sequences sufficient to direct the synthesis and secretion of foreign proteins (14). The *Eco*RI to *Hind*III  $\alpha$ -factor fragment was obtained by cloning the  $\alpha$ -factor gene using as a probe a synthetic oligonucleotide derived from published sequences (15). The *Sfa*NI–*Nco* I fragment of pHG23 (see Fig. 2A) was fused in-frame to the  $\alpha$ -factor signal sequence by use of a synthetic oligonucleotide (Fig. 1). This oligonucleotide linker joins the *Hind*III site of  $\alpha$  factor to the *Sfa*NI site of GM-CSF, adding a second  $\alpha$ -factor processing site to obtain complete processing of the signal (14) for secretion of the mature form of GM-CSF.

**Abbreviations:** CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; Con A, concanavalin A; PMA, phorbol myristate acetate; bp, base pair(s).

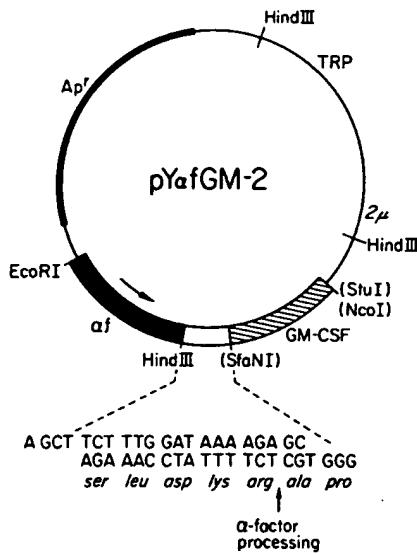


FIG. 1. Structure of the yeast expression plasmid. The plasmid, pYafGM-2, contains sequences derived from pBR322 (thick line) containing the origin of replication and ampicillin resistance gene (Ap') and sequences from yeast (thin line) including the *TRP-1* gene as a selectable marker and the 2-μm origin of replication. The solid box indicates the α-factor (af) promoter and leader sequences used to direct transcription and secretion of GM-CSF. The synthetic oligonucleotides used to fuse the α-factor sequences with GM-CSF sequences (hatched box) are shown.

The expression plasmid was transformed into yeast strain 79 [ $\alpha$ , *trp1-1*, *leu2-1*] selecting for  $\text{Trp}^+$  transformants. Cultures to be assayed for biological activity were grown in 20–50 ml of rich media (1% yeast extract/2% peptone/2% glucose) at 30°C to stationary phase. The protease inhibitors phenylmethylsulfonyl fluoride and pepstatin A were added at the time of harvest to a final concentration of 1 mM and 10  $\mu\text{M}$ , respectively. Cells were then removed by centrifugation, and the medium was filtered through a 0.45-μm cellulose acetate filter.

**Preparation of Human Bone Marrow Cells.** Human bone marrow from the iliac crest of healthy donors was collected in a heparinized syringe. The marrow was diluted 1:3 with phosphate-buffered saline at room temperature and layered onto a solution of 54% Percoll (Pharmacia). After centrifugation at  $500 \times g$  at room temperature for 20 min, the interface was collected and washed with 20 vol of phosphate-buffered saline. The suspension was then centrifuged at  $250 \times g$  for 10 min. Cells were resuspended in the desired volume of alpha minimal essential medium (MEM  $\alpha$  medium) with nucleotides (GIBCO) for cell counting and viability determination. Serum was then added, and the cell suspension was stored on ice until assay.

**Colony Assay.** The presence of human GM-CSF was determined by its ability to stimulate the growth of colonies in agar. Bone marrow cells were added at a final concentration of  $1 \times 10^5$  per ml to incubation medium consisting of 3 parts of a 1.4% bacto-agar solution (Difco) and 7 parts of a solution containing 28.1% fetal calf serum, 70  $\mu\text{M}$  mercaptoethanol, 0.12 mg of asparagine and 0.7 mg of glutamine per ml, 150 units of penicillin G, 150 units of streptomycin, 1.1× MEM  $\alpha$  medium with nucleotides and 2.2× vitamins (GIBCO). The cultures were incubated in humidified 5%  $\text{CO}_2$ /95% air at 37°C and examined after 7 and 14 days to determine the number and type of colonies (granulocyte, macrophage, and mixed granulocyte/macrophage).

**RNA Analysis.** Primer extension experiments were performed as described (8) except that electrophoresis was in a 6% polyacrylamide/8 M urea gel.

Total RNA for Northern blots was isolated by the guanidinium thiocyanate/cesium chloride method (16). Samples were then electrophoresed in 1.1% agarose gels containing formaldehyde, transferred to nitrocellulose filters (16), and hybridized with a  $^{32}\text{P}$ -labeled RNA probe transcribed by SP6 polymerase (17). The  $^{32}\text{P}$ -RNA probe was synthesized from the 600-base-pair (bp) *Pst* I to *Nco* I fragment of pHG23, which was inserted into pSP64 (Promega Biotec, Madison, WI). Hybridization and washing of blots was as described (8).

## RESULTS

**Cloning and Sequencing of Mouse and Human GM-CSF DNA.** A cDNA library was constructed with polyadenylylated messenger RNA from the mouse T-cell lymphoma line LBRM-33-5A4 stimulated with phytohemagglutinin. An oligonucleotide complementary to the 40 nucleotides at the 5' end of the published sequence for mouse GM-CSF (6) was synthesized, labeled with  $^{32}\text{P}$ , and used as a hybridization probe to screen the library. One hybridizing clone, TH1, was isolated and sequenced (Fig. 2B). This sequence contained a single, long, open-reading frame beginning 27 nucleotides 5' of the previously published sequence for mouse GM-CSF and largely identical to it except for a few nucleotide changes. The two sequences will be compared elsewhere (unpublished data). The cDNA of TH1 has been shown to direct production of biologically active GM-CSF using a yeast expression system (unpublished results).

A 372-bp *Hae* III fragment from TH1, extending from nucleotide positions 42 to 414 (Fig. 2B) was isolated, nick-translated, and used under conditions of low stringency to probe two human cDNA libraries that were constructed from mRNA isolated from either the human cell line HUT-102 or from mitogen-stimulated human peripheral blood T lymphocytes. A number of cDNA clones that contained sequences complementary to the mouse GM-CSF probe were isolated from these libraries and shown by cross-hybridization and restriction mapping to be related. The cDNA from two of these HUT-102 clones (pHG23 and pHG25) is shown in Fig. 2A. Sequence analysis of these clones showed an open-reading frame encoding a protein of 144 amino acids with a predicted molecular mass of 16,293 daltons. The cDNA includes the predicted amino acid sequence Ala-Pro-Ala-Arg-Ser-Pro-Ser-Pro-Ser, which is identical to the reported amino terminus for mature human GM-CSF protein (5). The 5' end of the open-reading frame encodes a region of 17 amino acids, with a potential initiator methionine and many of the characteristics expected of a hydrophobic signal peptide for a secreted protein (18).

**Synthesis of Recombinant GM-CSF in Yeast.** To show that the cDNA in pHG23 encoded a protein with GM-CSF activity, we constructed a plasmid designed to direct synthesis and secretion of the mature form of GM-CSF from yeast (see Fig. 1). Secretion of the mature protein is expected to result in glycosylation of the protein. The construction contains the yeast α-factor promoter plus leader sequences sufficient to direct the secretion of GM-CSF, followed immediately by the sequence for mature GM-CSF beginning with the alanine at amino acid position 1 in Fig. 2B. Supernatants from yeast transformed with the GM-CSF expression plasmid or from the control plasmid, pYaf, lacking the GM-CSF sequences of Fig. 1, or from human placental cells cultured at  $1.2 \times 10^7$  per ml for 6 days in the presence of 5% fetal bovine serum were assayed for the ability to direct the formation of mixed granulocytic and macrophage-type colonies from human bone marrow cells as described. pYafGM-2 directed synthesis of high levels of GM-CSF activity, where-

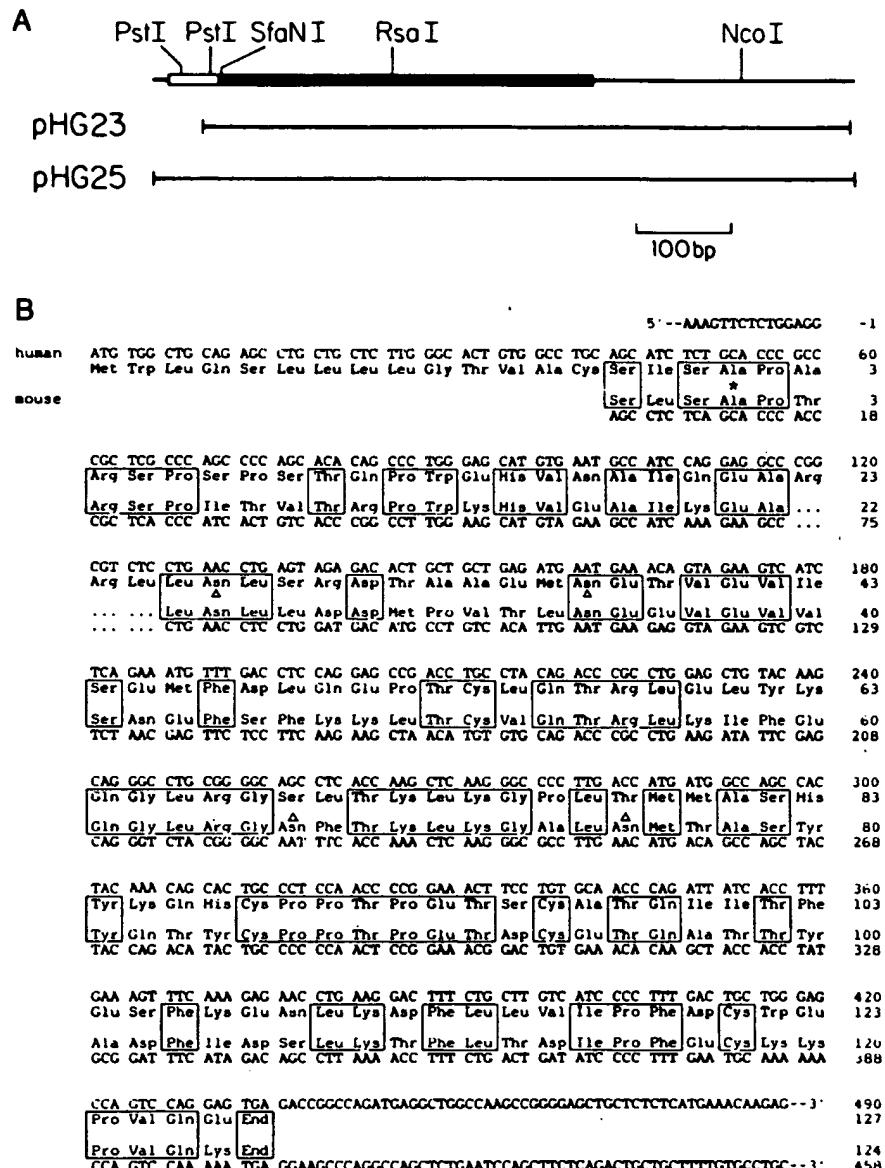


FIG. 2. Restriction map and nucleotide sequence of GM-CSF cDNA clones. (A) Partial restriction map of human GM-CSF cDNA. Coding sequences are boxed: the open box represents the proposed signal sequence and the shaded box represents the coding region for mature protein. The sizes of the cDNA inserts in pHG23 and pHG25 are indicated. (B) The nucleotide sequences and the predicted amino acid sequences for mouse and human GM-CSF. The cDNA sequences of the complete 3' untranslated regions are not shown. The nucleotides are numbered from the presumed initiator methionine codon in the human GM-CSF and from the first nucleotide in the mouse GM-CSF. The amino acids are numbered from the amino terminus of the mature proteins, the alanine residues marked with a star. Boxed amino acid residues indicate regions of homology between the mouse and human proteins. Triangles represent possible N-linked glycosylation sites.

as the control plasmid, pYaf, produced supernatants with no activity. The titer [granulocytic progenitor cells (CFU-C) per ml] was calculated by multiplying by 50 the reciprocal of the dilution giving 50% of the maximum colony number: pYaf/Gm-2,  $1.25 \times 10^6$  CFU-C per ml; pYaf, 0 CFU-C per ml; placental cells,  $5 \times 10^2$  CFU-C per ml. (In seven experiments with different donors, the average maximum number of colonies from  $10^5$  marrow cells was  $96 \pm 29$ .) At 14 days, the recombinant GM-CSF and placental cell supernatants each produced well-defined colonies, of which approximately one-third was granulocyte/macrophage colonies, one-third was dispersed macrophage colonies, and one-third was tight granulocyte colonies. We have observed the same types of

colonies with recombinant murine GM-CSF in murine bone marrow colony assays (unpublished data).

**Analysis of mRNA.** Since the availability of cells that synthesize GM-CSF is limited, we investigated the expression of GM-CSF mRNA from a number of cell types that have been reported previously to express other CSFs. Blots of RNA from these cells were analyzed by hybridization with a probe derived from pHG23. Fig. 3 *Left* shows strong hybridization to a band of approximately 900 nucleotides in RNA from peripheral blood T cells activated with PMA and Con A and from HUT-102 cells (lanes 3 and 1). A low level of hybridization was seen in RNA from a human bladder tumor cell line (lane 6) and no hybridization was seen in RNA from

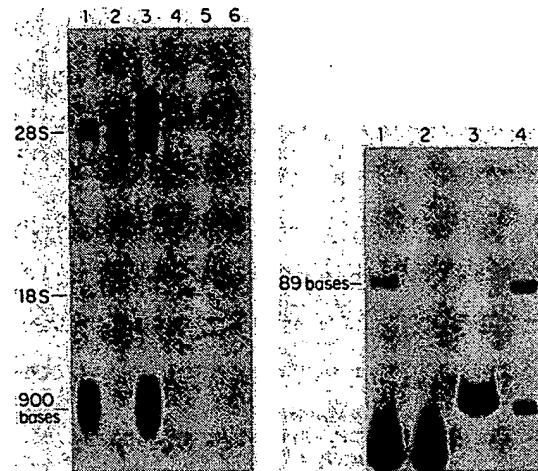


FIG. 3. Analysis of GM-CSF messenger RNA. (Left) Hybridization of GM-CSF probe to blots of RNA from human cells. Lanes: 1, 5  $\mu$ g of total RNA from HUT-102 cells; 2, 5  $\mu$ g of total RNA from unstimulated peripheral blood T cells; 3, 5  $\mu$ g of total RNA from peripheral blood T cells stimulated with Con A and PMA; 4, 1.5  $\mu$ g of polyadenylated RNA from peripheral blood macrophages stimulated with lipopolysaccharide; 5, 1.5  $\mu$ g of polyadenylated RNA from the pancreatic carcinoma cell line 1420; 6, 1.5  $\mu$ g of polyadenylated RNA from the bladder carcinoma cell line 5637. The positions of 18S and 28S rRNA bands are indicated. (Right) Primer extension analysis of GM-CSF RNA using a 5' end-labeled probe complementary to the mRNA from nucleotide numbers 38–57 in Fig. 2B. Lanes: 1, analysis of 30  $\mu$ g of total RNA from peripheral blood T cells stimulated with Con A and PMA; 2, analysis of 30  $\mu$ g of total RNA from the Jurkat T-cell lymphoma cell line; 3, a 47-base oligonucleotide that was 5' end-labeled with  $^{32}$ P as a size marker; 4,  $^{32}$ P-labeled size markers of 88 and 42 bp derived from an *Ava* II digest of pBR322.

unstimulated T cells, lipopolysaccharide-stimulated macrophages, and a human pancreatic tumor cell line (lanes 2, 4, and 5). Other, higher molecular weight bands in Fig. 3 Left are due to hybridization of the probe to ribosomal RNA.

To determine the position of the 5' end of the GM-CSF mRNA, primer extension experiments were performed by using an oligonucleotide complementary to the mRNA from nucleotide numbers 38–57 in Fig. 2B. A single primer extension product of 89 nucleotides was seen with RNA from mitogen-stimulated T cells (Fig. 3 Right), indicating that the cDNA of pHG25 is approximately 17 bp short of the 5' end of the natural mRNA. Therefore, this mRNA must have an extremely short 5' untranslated region. No primer extension product was seen with RNA from the T-cell lymphoma line Jurkat.

**Analysis of Genomic Sequences.** To determine the number of GM-CSF-related genes in human DNA, we hybridized a  $^{32}$ P-labeled human GM-CSF probe to Southern blots of human DNA digested with a number of restriction enzymes expected to cut relatively infrequently. The results (Fig. 4) show that digestion with *Hind*III, *Eco*RI, or *Pst* I gave rise to a single band, while digestion with *Bgl* II gave rise to two bands. On the basis of these results, it appears probable that the gene for GM-CSF exists as a single-copy gene.

## DISCUSSION

In this study we have used a murine GM-CSF cDNA probe to isolate a homologous sequence from human cDNA libraries. Two lines of evidence support the assumption that this sequence corresponds to the human GM-CSF gene. The amino acid sequence of the protein predicted from our

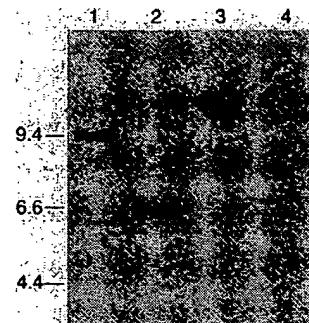


FIG. 4. Hybridization of GM-CSF cDNA to Southern blots of human genomic DNA. Genomic DNA (10  $\mu$ g) was digested with *Hind*III (lane 1), *Eco*RI (lane 2), *Pst* I (lane 3), or *Bgl* II (lane 4), electrophoresed in a 0.7% agarose gel, blotted, and hybridized at high stringency to nick-translated  $^{32}$ P-labeled GM-CSF cDNA by standard techniques. The molecular weight markers (in kilobase pairs) are from *Hind*III-digested bacteriophage  $\lambda$  DNA.

nucleotide sequence includes nine amino acids that are identical to those reported by Gasson *et al.* (5) for the amino terminus of naturally occurring human GM-CSF. In addition, we have constructed an expression plasmid designed to direct the synthesis and secretion of this protein by yeast and have shown that it can stimulate the growth of granulocyte, macrophage, and granulocyte/macrophage colonies from human bone marrow.

In contrast, the secreted, recombinant human GM-CSF showed no activity in three biological assays that respond to recombinant murine GM-CSF. These are: a murine bone marrow colony assay, a murine bone marrow proliferation assay, and a murine bone marrow macrophage proliferation assay. Similarly, recombinant murine GM-CSF has shown no activity in human bone marrow assays (unpublished data). This lack of cross-species activity contrasts to that reported for human macrophage CSF, which can stimulate the growth of murine macrophages (19).

The human GM-CSF nucleotide sequence reported here predicts a molecular mass for the mature polypeptide of 14,476 daltons, starting at the position corresponding to the published amino terminus (position 1 in Fig. 2B). The naturally occurring glycoprotein purified from the Mo T-lymphoblast cell line is reported to have a molecular mass of 22,000 daltons (5), indicating that it could contain up to 34% carbohydrate by weight. Potential N-linked glycosylation sites exist at asparagine residues 27 and 37. Interestingly, the sequence of murine GM-CSF also contains two potential N-linked glycosylation sites, but in a different region of the molecule (asparagine residues 66 and 75). Comparison of the human and mouse sequences reveals substantial homology at both the nucleotide (69%) and amino acid (54%) levels throughout the coding regions. The amino terminus of murine GM-CSF has been reported to be Ile-7, whereas sequence comparison with human GM-CSF would suggest Ala-1 as a more likely possibility. The position of the carboxyl terminus of the two proteins is identical, as are the positions of the four cysteine residues, indicating the possible importance of disulfide bond formation in these molecules (5). The human sequence has an insertion of nine nucleotides relative to the mouse sequence, giving rise to three additional amino acids at positions 23–25.

The assignment of the initiator methionine at position –17 must be considered as preliminary in the absence of any upstream, in-phase, termination codons or of the full-length cDNA sequence. However, as we have sequenced to within 17 nucleotides of the 5' end of the mRNA, and the signal sequence predicted here conforms well to those reported for

other secreted and membrane proteins, it is probable that we have identified the true initiator methionine.

We have shown that HUT-102 cells and peripheral blood T cells activated with PMA and Con A contained relatively high amounts of GM-CSF mRNA (Fig. 3 Left). It has previously been reported that HUT-102 cells produce a CSF (20). Interestingly, a human bladder carcinoma cell line (5637), reported to make an early hematopoietic factor (21), showed an abundance of GM-CSF mRNA. However, analysis of mRNA from the human pancreatic cell line (1420), which has been reported to make human macrophage CSF and possibly GM-CSF (22), produced no detectable GM-CSF mRNA.

The availability of recombinant human GM-CSF should now allow unequivocal analysis of the physiological role of GM-CSF in hematopoiesis and immune functions.

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# P- and E-Selectin Use Common Sites for Carbohydrate Ligand Recognition and Cell Adhesion

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**Abstract.** The selectins are a family of three calcium-dependent lectins that mediate adhesive interactions between leukocytes and the endothelium during normal and abnormal inflammatory episodes. Previous work has implicated the carbohydrate sialyl Lewis<sup>x</sup> (sLe<sup>x</sup>; sialic acid alpha 2-3 galactose beta 1-4 [Fucose alpha 1-3] N-acetyl glucosamine) as a component of the ligand recognized by E- and P-selectin. In the case of P-selectin, other components of the cell surface, including 2'6-linked sialic acid and sulfatide (galactose-4-sulfate ceramide), have also been proposed for adhesion mediated by this selectin. We have recently defined a region of the E-selectin lectin domain that appears to be directly involved with carbohydrate rec-

ognition and cell adhesion (Erbe, D. V., B. A. Wolitzky, L. G. Presta, C. R. Norton, R. J. Ramos, D. K. Burns, R. M. Rumberger, B. N. N. Rao, C. Foxall, B. K. Brandley, and L. A. Lasky. 1992. *J. Cell Biol.* 119:215-227). Here we describe a similar analysis of the P-selectin lectin domain which demonstrates that a homologous region of this glycoprotein's lectin motif is involved with carbohydrate recognition and cell binding. In addition, we present evidence that is inconsistent with a biological role for either 2'6-linked sialic acid or sulfatide in P-selectin-mediated adhesion. These results suggest that a common region of the E- and P-selectin lectin domains appears to mediate carbohydrate recognition and cell adhesion.

**P**- and E-selectin are calcium-dependent cell surface lectins that mediate leukocyte adhesion by recognition of cell-specific carbohydrate ligands. Together with L-selectin, they form a family of cell adhesion molecules (24, 25) that contain lectin, epidermal growth factor (EGF)-like, and complement binding-like domains (4, 18, 28, 36, 39). L-selectin is found on all leukocytes and appears to recognize an unknown sialylated, fucosylated, sulfated carbohydrate ligand on at least two endothelial glycoproteins (17, 42). One of these glycoproteins, which has been named GlyCAM 1, is a mucin-like endothelial glycoprotein that appears to present carbohydrate ligands to the L-selectin lectin domain (27). E-selectin is an endothelial adhesion molecule whose expression is induced by various inflammatory stimuli and which recognizes the cell surface carbohydrate, sialyl Lewis x (sLe<sup>x</sup>); sialic acid alpha 2-3 galactose beta 1-4 [fucose alpha 1-3] N-acetyl glucosamine) (29, 33, 40, 45). P-selectin is found stored in alpha granules of platelets as well as Weible-Palade bodies of endothelial cells (5, 30). It appears to recognize a carbohydrate that is either identical, or closely related, to sLe<sup>x</sup> (22, 23, 31, 34) in the context of perhaps a single glycoprotein (31, 32, 37).

1. Abbreviations used in this paper: Fc<sub>γ</sub>R, IgG Fc receptors; MBP, mannose binding protein; sLe<sup>x</sup>, sialyl Lewis x.

Much evidence has accumulated to indicate similarities in the nature of the carbohydrates seen by selectins. Sialylated, fucosylated lactosaminoglycans (such as sLe<sup>x</sup>) have been shown to bind L-, E-, and P-selectin (12, 16, 29, 33, 34, 40, 45, 49). Furthermore, all three selectins require both sialic acid and fucose residues in specific linkages for adhesion (7, 8, 38, 43). However, the exact carbohydrate structures recognized by selectins are currently incompletely characterized. In addition, many studies have demonstrated clear differences in carbohydrate recognition by selectins. For example, in contrast to E-selectin, both L- and P-selectin have been shown to bind sulfatide (galactose-4-sulfate ceramide) (1, 12). Another distinction in selectin-carbohydrate interactions emerged in a recent study by Larsen and colleagues (24) in which the *Sambucus nigra* lectin, which is specific for the sialyl-2'6 $\beta$ Gal/GalNAc linkage, blocked P-selectin binding but not E-selectin binding. This led these authors to propose that the carbohydrate recognized by P-selectin contains a terminal 2'3 sLe<sup>x</sup> core plus a second, perhaps branched terminal sialic acid linked 2'6 to a galactose, whereas the carbohydrate recognized by E-selectin may be simply 2'3 sLe<sup>x</sup> (24). In fact, P-selectin is the only selectin that binds to the 2'6 form of an sLe<sup>x</sup> glycolipid immobilized on microtitre wells, although this binding is weak and variable (12). Thus, evidence exists for the possible participation

of sulfatides and/or 2'6-linked sialic acid in conferring specificity in P-selectin binding to its ligand. Specificity in selectin-carbohydrate interactions may stem from other variations on this common carbohydrate (i.e., sLe<sup>x</sup>-like) theme, such as the addition of sulfate for the L-selectin ligand (Imai, Y., L. A. Lasky, and S. Rosen, manuscript submitted for publication). Additionally, specificity may stem from variations in carbohydrate presentation (44). For instance, specific glycoproteins appear to present the L- and P-selectin cognate carbohydrate ligands whereas E-selectin appears to recognize a glycolipid (or a protease-resistant glycoprotein) (28, 32).

Because of these differences in carbohydrate recognition within the context of structural similarities, it seems reasonable to suppose that the highly homologous lectin domains of selectins may use a common recognition site for sugars, with some interspersed amino acid variation that confers specificity. Previously, we used a combination of mutagenesis and modeling to identify a discontinuous region of the E-selectin lectin domain that appears to form at least part of its binding site for 2'3 sLe<sup>x</sup> (10). This study modeled the E-selectin lectin domain using the crystal structure of the mannose binding protein (47) as a guide and demonstrated the importance of residues within a patch, formed by the antiparallel beta sheet derived from the amino and carboxy termini and two adjacent loops, in the binding of both blocking anti-E-selectin mAbs, and in the binding of sLe<sup>x</sup> itself (10). Additionally, the relatively small size of the E-selectin region identified as critical for ligand recognition was consistent with nuclear magnetic resonance solution analyses of 2'3 sLe<sup>x</sup> structure showing that the critical sialic acid and fucose residues point to one face of this carbohydrate and are separated by ~10 Å (2, 43).

In this study we extend understanding of selectin structure and function by demonstrating that the site identified as important for E-selectin binding also serves an important role in P-selectin binding. Mutagenesis of this site in P-selectin also provides clues to some of the structural basis for the similarities and differences in carbohydrate recognition by selectins.

## Materials and Methods

### Flow Cytometric Assay for P-selectin Ligand

The interaction of P-selectin and its cellular ligand was studied using a flow cytometric assay. Cells used in this assay were either HL60 cells (maintained in high glucose DME plus 10% Hyclone FBS) or fresh human neutrophils. Human neutrophils were purified from heparinized peripheral blood by a Ficoll-Hypaque gradient to remove mononuclear cells, followed by treatment with 3% dextran sulfate to remove red blood cells. The resulting cells were >90% neutrophils. Before staining with P-selectin-IgG both cell types were preincubated in Dulbecco's PBS/1% BSA/0.1% sodium azide/1% normal rabbit serum (staining medium) for 30–60 mins on ice. After this initial incubation, 1 µg of P-selectin-IgG was added to 100-µl aliquots of 10<sup>6</sup> cells and incubated for 30–60 mins on ice. The cells were then washed with staining medium and resuspended in 100 µl of staining medium to which was added 2 µl of a phycoerythrin-conjugated F(ab)<sub>2</sub> goat anti-human IgG (Fc specific). The cells were incubated for 15–30 mins on ice, washed twice with staining medium, and resuspended in 0.5 ml of staining medium before flow cytometric analysis on a FACScan (Becton Dickinson & Co., Mountain View, CA). To determine that the staining was an interaction of P-selectin with its ligand, the staining was also done in the presence of 10 mM EGTA. To determine the protease sensitivity and the requirement for sialic acid of this interaction, HL-60 cells in D-PBS and 1% BSA were incubated with either trypsin or Arthrobacter or Clostridium

sialidases at 37°C before resuspending in staining medium. To determine the effect of activation on the expression of the ligand, human neutrophils were incubated at 37°C with 50 ng/ml phorbol myristate acetate for 10 min before resuspending in staining medium. To examine the ability of various carbohydrates to inhibit staining, 50 µg/ml fucoidin (Sigma Immunochemicals, St. Louis, MO), 50 µg/ml dextran sulfate (Pharmacia Fine Chemicals, Piscataway, NJ), 10 mg/ml mannose-1-phosphate (Sigma Immunochemicals), or 10 mg/ml mannose-6-phosphate (Sigma Immunochemicals) was added to cells immediately before the addition of the P-selectin chimera. Each carbohydrate was then present until the cells were washed before the addition of the second stage antibody. A potential complication of this FACS assay arose from the use of selectin-IgG chimeras to stain cells (HL60 cells and neutrophils) which bear human IgG Fc receptors (Fc<sub>Y</sub>R; see reference 11 for review). Adding rabbit IgG (in the form of normal rabbit serum) to the assay medium blocked this binding in most cases. However, in some experiments with human neutrophils, it was necessary to add murine mAbs to human Fc<sub>Y</sub>R (Medarex, Inc., West Lebanon, NH) to the assay medium to completely block this interaction.

### Anti-selectin mAbs

The following anti-human P-selectin mAbs were purchased to characterize the mutant chimeras: mAbs AK-6 (CLB-thromb6) and CRC 81 from Bio-Design International (Kennebunkport, ME), and mAb AC 1.2 from Becton Dickinson & Co. The anti-E-selectin mAbs 9A1, 7E10, 3B7, and 9H9 have been described (10). The anti-L-selectin mAb Leu 8 was purchased from Becton Dickinson & Co. and used in the FACS assay (a registered trademark of Becton Dickinson & Co.) according to the manufacturer's instructions.

### Construction and Expression of Wild Type and Mutant Chimeras

Production and characterization of the P- and E-selectin-IgG chimeras has been previously described (12). The PE-1 chimera was constructed in two steps. First, an EcoRI-Xhol fragment encoding the signal peptide, lectin domain, and part of the EGF domain of P-selectin was removed from a pRKS/P-selectin-IgG plasmid. This fragment was inserted into a pRKS/E-selectin-IgG plasmid which had been digested with EcoRI and BgIII to remove the E-selectin signal peptide and most of the E-selectin lectin domain. Second, the P-selectin lectin domain was joined in frame to the E-selectin EGF domain via oligonucleotide-directed deletional mutagenesis using the method of Kunkel (21) as described (10). The expressed PE-1 construct consisted of the signal peptide and lectin domain from P-selectin, followed by the EGF, C1, and C2 domains of E-selectin, and the IgG1 hinge, CH2 and CH3 domains common to both the P-selectin-IgG and E-selectin-IgG constructs.

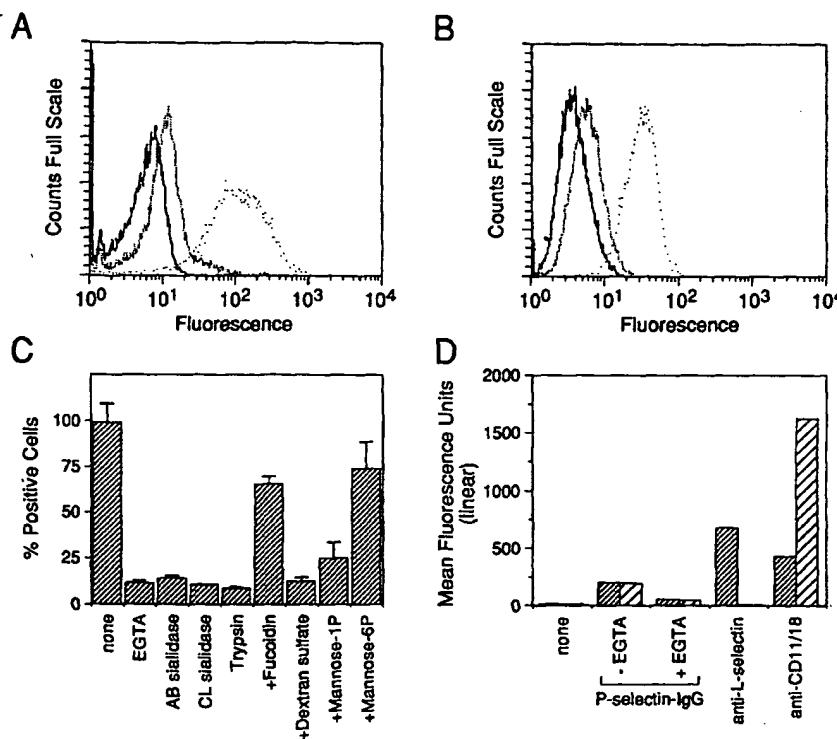
Amino acid substitutions were introduced into the lectin domain of the P-selectin-IgG chimera as previously described (10). Wild type and mutant chimeras were expressed and secreted by 293 cells, quantified and tested for anti-selectin mAb reactivity also as described (10). Mutant chimeras are defined using the nomenclature: K113A is a mutant where the lysine (K) at position 113 is changed to an alanine (A).

### Binding of Selectin-IgG Chimeras to Sialyl Lewis x and Sulfatides

Assays for binding of the different selectin-IgG chimeras to immobilized sLe<sup>x</sup> glycolipids or sulfatides were performed as described (12). Briefly, 23 sLe<sup>x</sup> glycolipids, 26 sLe<sup>x</sup> glycolipids, or bovine brain sulfatides (Sigma Immunochemicals) were dried onto microtitre wells, washed with distilled water, and then blocked with BSA. Biotinylated goat anti-human IgG Fc and alkaline phosphatase-streptavidin (Caltag Labs, South San Francisco, CA) were each diluted 1:1,000 into 293 cell supernatants containing equal concentrations of wild type or mutant chimeras and allowed to form a complex before the addition to the wells. These supernatants were then incubated on the sLe<sup>x</sup> glycolipid or sulfatide-coated surfaces, followed by washing, addition of substrate (*p*-nitrophenyl phosphate), and measurement of the OD at 405 nm.

### Generation of a P-selectin Lectin Domain Model

A model of the P-selectin lectin domain was generated based on the crystal structure of the rat mannose-binding protein (MBP) (47) as previously described for an E-selectin lectin domain model (10). Briefly, MBP residues were changed to the P-selectin sequence with the sidechain conformations



**Figure 1.** Staining of HL60 cells and neutrophils by selectin-IgG chimeras. Selectin-IgG chimeras were tested by flow cytometry for staining of either HL60 cells (*A* and *C*) or human neutrophils (*B* and *D*) as described in Materials and Methods. In *A* and *B*, dotted lines represent P-selectin-IgG staining, dashed lines represent P-selectin-IgG staining in the presence of 10 mM EGTA, and solid lines represent E-selectin-IgG staining. Staining with no chimera (secondary antibody only) was identical to E-selectin-IgG staining for both cell types. (*C*) HL60 cells were treated as indicated (see Materials and Methods) and stained with P-selectin-IgG as in *A*. Results are expressed as percentage of cells staining positively ( $\pm$ SD of duplicates) based on staining with secondary antibody alone. (*D*) Human neutrophils before (■) or after (▨) activation with PMA were stained with the indicated reagents and evaluated by flow cytometry as described in Materials and Methods. Results are shown as the linear mean fluorescence.

kept similar to those of MBP where possible. Otherwise sidechain conformations were based on rotamer libraries (35), packing, and hydrogen-bonding considerations. Possible loop structures for the 11 insertions and two deletions in P-selectin relative to MBP were gleaned from a search of crystal structures in the Protein Data Bank (3). Finally, the P-selectin model was subjected to repetitive cycles of energy minimization using the method described for E-selectin (10).

## Results

As a starting point for evaluating the residues in P-selectin responsible for binding ligand, we developed a flow cytometric assay using the P-selectin-IgG chimera to stain HL60 cells and neutrophils. Whereas E-selectin-IgG did not bind HL60 cells or neutrophils in this assay, P-selectin-IgG staining resulted in a strong fluorescence shift for both cell types (Fig. 1, *A* and *B*). This binding was inhibited by EGTA, reflecting the calcium requirement for P-selectin's interaction with its ligand. Further controls indicated that this assay using the P-selectin-IgG chimera reflects the published characteristics of P-selectin-ligand binding (8, 24, 31, 32, 37, 49). In particular, treatment of HL60 cells with either trypsin or sialidase abolished staining (Fig. 1 *C*). Furthermore, P-selectin-IgG staining was inhibited by dextran sulfate and mannose-1-phosphate, but not by fucoidin or mannose-6-phosphate (Fig. 1 *C*). After activation of human neutrophils with PMA, although surface expression of L-selectin decreased and surface expression of CD11/18 increased, surface expression of the P-selectin ligand did not change (Fig. 1 *D*). In addition to neutrophils, monocytes and NK/LGL cells were positive when stained with P-selectin-IgG (data not shown), which is consistent with the expression of the P-selectin ligand on these cells.

As noted above, the E-selectin-IgG chimera did not bind

HL60 cells or neutrophils in the soluble FACS assay. We exploited this finding to aid in mapping the region of P-selectin necessary for conferring this high affinity binding. Since our previous study with E-selectin (10) had localized its ligand binding site to a region within its lectin domain, we sought to determine if the apparent differences in E- and P-selectin binding could be attributed to differences in their lectin domains. Consequently, we constructed a chimera (PE-1) which consisted of E-selectin-IgG with the E-selectin lectin domain replaced with the lectin domain from P-selectin. To see if this chimera was folded correctly, we tested its binding to antibodies specific for the various domains of E- and P-selectin. The PE-1 chimera reacted well with antibodies to the CRI and CR2 domains of E-selectin (mAbs 9A1 and 7E10, Table I) but not with antibodies to the lectin domain of E-selectin (mAbs 3B7 and 9H9, Table I). PE-1 bound to the blocking antibody to P-selectin (9) (AK-6, Table I), consistent with the localization of the epitope recognized by this mAb to the lectin domain of P-selectin. By contrast, the non-blocking antibodies to P-selectin, AC 1.2 and CRC 81, did not recognize PE-1 (Table I). This latter result is consistent with earlier studies which indicated a contribution of residues within the EGF and/or CR domains of P-selectin in AC 1.2 binding (19). These results are consistent with the PE-1 chimera being correctly folded, and indicate that at least part of the epitope recognized by the blocking mAb AK-6 is localized to the lectin domain of P-selectin.

To determine if transferring the P-selectin lectin domain onto E-selectin-IgG transferred carbohydrate specificity, we examined binding of PE-1 to various immobilized glycolipids. This binding was compared to that seen with either P-selectin-IgG or E-selectin-IgG. As shown in Fig. 2, the PE-1 chimera appeared to closely mimic P-selectin-IgG in

**Table I. PE-1 Binding to Anti-selectin Antibodies**

mAb Class	Clone	P-selectin-IgG	OD 450 PE-1	E-selectin-IgG
Blocking mAb to P-selectin	AK-6	1.3 ± 0.1	1.7 ± 0.1	0.0 ± 0.0
Nonblocking mAb to P-selectin	AC 1.2	2.8 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
	CRC 81	3.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
mAb to CR1 and CR2 of E-selectin	9A1	0.1 ± 0.0	1.1 ± 0.2	0.9 ± 0.1
	7E10	0.0 ± 0.0	1.6 ± 0.0	1.2 ± 0.2
mAb to lectin domain of E-selectin	3B7	0.1 ± 0.1	0.0 ± 0.0	1.9 ± 0.0
	9H9	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 0.1

The P-selectin-IgG, E-selectin-IgG, and PE-1 chimeras were tested for capture by the antibodies indicated using the ELISA format described previously (10). Results shown are the mean optical density ± SD of duplicate determinations.

binding to all three glycolipids tested: 2'3 sLe<sup>a</sup> (Fig. 2 A), 2'6 sLe<sup>a</sup> (Fig. 2 B) and sulfatides (Fig. 2 C). Therefore, the lectin domain of P-selectin appears to be sufficient for transferring specificity in binding to these purified glycolipids.

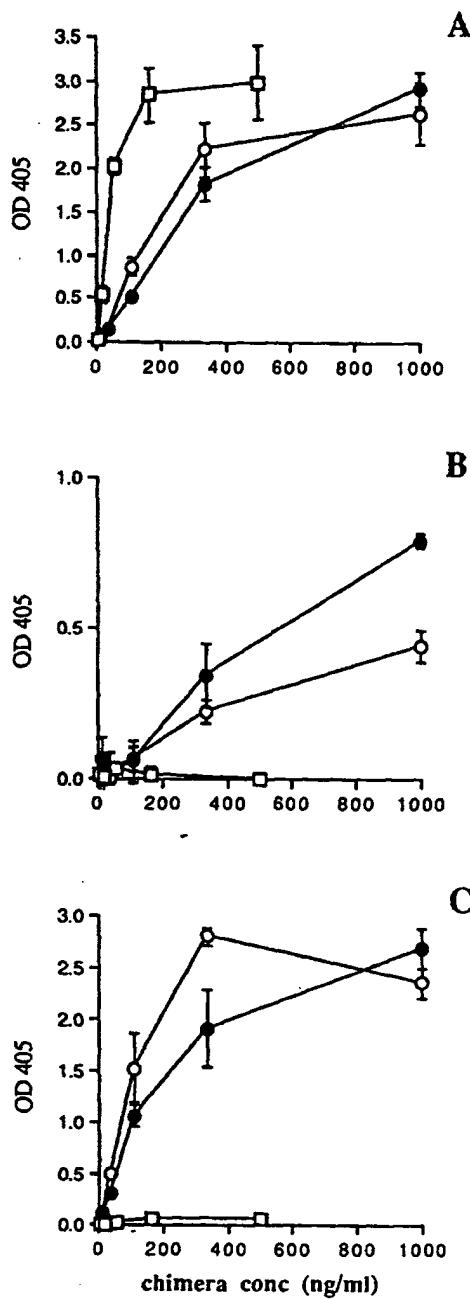
We then tested the PE-1 chimera for cell staining to see if the P-selectin lectin domain could also confer the high affinity binding to the P-selectin ligand on HL60 cells. As seen in Fig. 3, the PE-1 chimera did bind HL60 cells. However, the shift in fluorescence seen with PE-1 staining was not as great as that seen with P-selectin-IgG (Fig. 3). Therefore, although the lectin domain of P-selectin did appear to clearly confer HL60 cell staining, some contribution of the EGF and/or CR1 domain of P-selectin may be required for full, high-affinity binding to these cells. Similar results were seen when neutrophils were stained with these three chimeras (data not shown).

The above results using the PE-1 chimera indicated that the lectin domain of P-selectin contained elements responsible for the differences in binding of E- and P-selectin to immobilized glycolipids and cells. Therefore, we performed mutagenesis of the P-selectin lectin domain to further localize the residues responsible for the interaction of P-selectin with its ligand. P-selectin mutagenesis was focused on those sites which in our previous study (10) proved to be important for E-selectin binding to its ligand. This strategy was followed for two reasons. First, as mentioned above, a wealth of experimental evidence exists indicating similarities in recognition of sugars by E- and P-selectin. Thus, it is reasonable to suppose that a site important for E-selectin-mediated adhesion would also participate in P-selectin-mediated binding. The second reason derived from an experimental consideration. In the E-selectin study we were able to generate an entire panel of antibodies to serve as structural controls for the effects of point mutations on lectin domain structure (10). This allowed the elimination of amino acid substitutions which grossly affected folding of the E-selectin lectin domain from consideration. In this study, we were limited to just three anti-P-selectin mAbs (AK-6, AC 1.2, and CRC 81), only one of which (AK-6) was clearly shown to bind a determinant in the lectin domain (see above). To avoid the generation and analysis of mutants which do not bind ligand due to a gross conformational effect rather than a specific side chain substitution, we restricted our analysis to only those mutations which had resulted in correctly folded proteins in the E-selectin analysis (10).

As a starting point for P-selectin mutagenesis we gener-

ated a three-dimensional model of the P-selectin lectin domain in the same manner that the E-selectin model was generated (see Fig. 4). Comparison of the two models revealed that of the residues that appeared most important for E-selectin binding to 2'3 sLe<sup>a</sup>, three are conserved in P-selectin: Y48, K111, and K113. In E-selectin, the substitutions Y48F, K111A, and K113A each profoundly decreased sLe<sup>a</sup> binding (10). Mutation of position 84 from R to A did not affect sLe<sup>a</sup> binding by E-selectin, and mutation of position 8 from E to A increased sLe<sup>a</sup> binding by E-selectin (10). Fig. 5 shows the effect of complementary substitutions at these positions in P-selectin on the binding of the anti-P-selectin mAbs. Whereas none of these substitutions significantly affected capture by the nonblocking antibodies (AC 1.2 and CRC 81), each of the substitutions K8A, K111A, and K113A partially decreased binding of the blocking antibody AK-6 (Fig. 5). These results are consistent with the PE-1 chimera results above which localized part of the AK-6 epitope to the lectin domain of P-selectin. These results are also consistent with the relatively close alignment of these three positions along the same face of the P-selectin lectin domain, as predicted by the model (Fig. 4). Furthermore, the complementary substitutions E8A and K113A in E-selectin completely abolished binding of a number of blocking mAbs to E-selectin (10). Also like E-selectin, mutation of the residues at positions 48 and 84 in P-selectin did not affect mAb binding (Fig. 5).

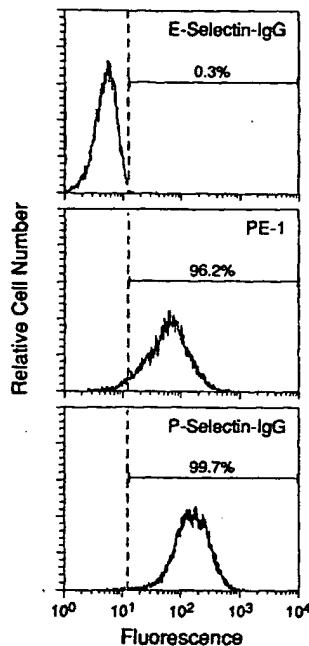
Next, we evaluated these P-selectin mutants for binding to immobilized glycolipids and cells (Fig. 6). Measurement of the binding of this panel of mutants to the 2'3 sLe<sup>a</sup> glycolipid indicated that P-selectin appears to use some of the same residues as E-selectin in binding this carbohydrate (Fig. 6 A). Whereas P-selectin mutants with the substitutions K8A and K84A still bound 2'3 sLe<sup>a</sup>, the mutants Y48F and K113A were completely negative. In E-selectin the mutant K111A did not bind 2'3 sLe<sup>a</sup> at all (10). Here, however, the P-selectin mutant K111A mediated partial binding to 2'3 sLe<sup>a</sup>, perhaps indicating a subtle difference in recognition of this sugar by E- and P-selectin. A different set of residues appeared to be important for binding of P-selectin to the 2'6 form of sLe<sup>a</sup> (Fig. 6 B). The substitutions K8A, K111A, and K113A ablated binding, while Y48F had no effect. The mutant K84A also still bound 2'6 sLe<sup>a</sup> (Fig. 6 B). When sulfatide binding was evaluated, a third pattern emerged (Fig. 6 C). Only the mutation K113A significantly decreased sulfatide binding by P-selectin. These results indicate that the



**Figure 2.** Binding of the PE-1 chimera to immobilized sLe<sup>X</sup> glycolipids and sulfatides. P-selectin-IgG (○), E-selectin-IgG (□), or PE-1 (●) were tested at the indicated concentrations for binding to immobilized 2'3 sLe<sup>X</sup> glycolipid (A), 2'6 sLe<sup>X</sup> glycolipid (B), or sulfatides (C) by the ELISA procedure described in Materials and Methods. Results shown represent the mean  $\pm$  SD of triplicate determinations.

same face of P-selectin appears to participate in binding these three glycolipids, with subtle differences in the residues used to bind each sugar.

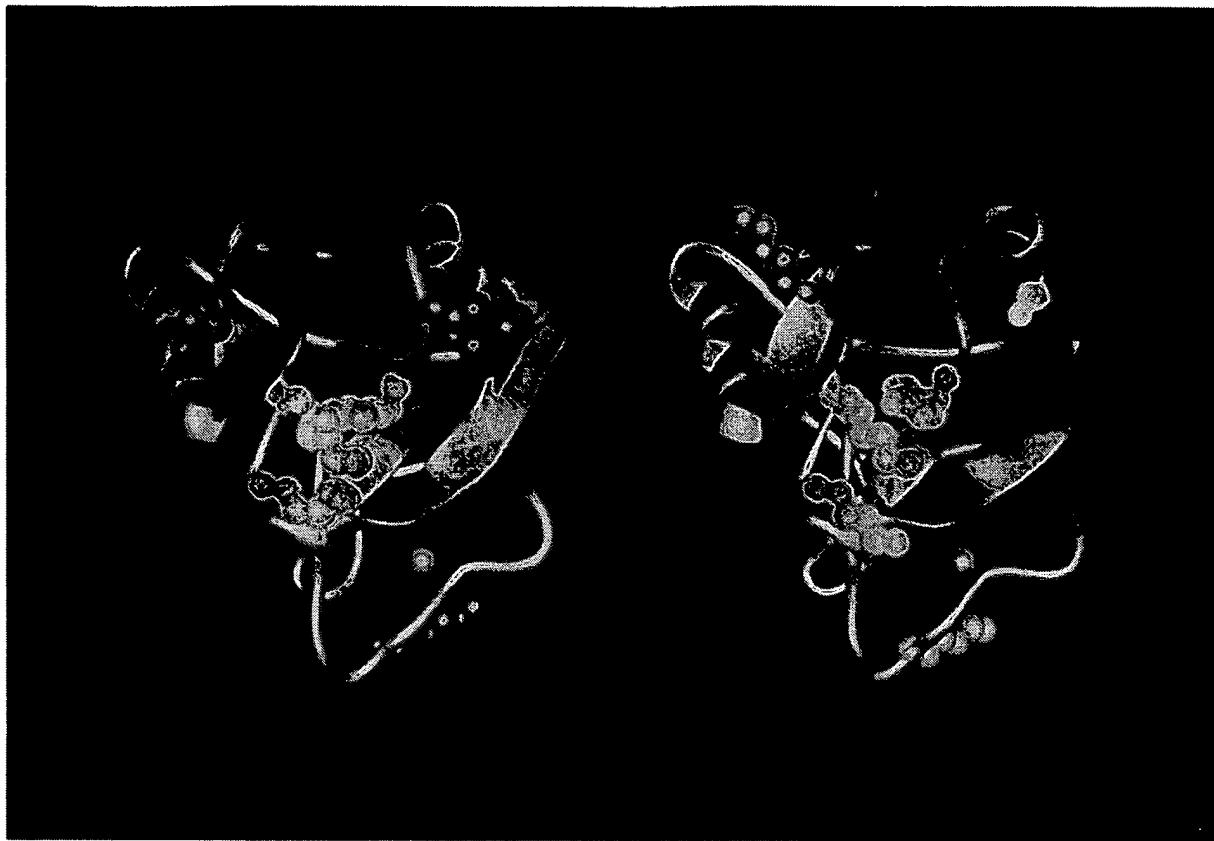
Since a more relevant assay for measuring P-selectin interactions with its ligand is the cell binding assay, the panel of mutants was evaluated by flow cytometry for staining of



**Figure 3.** Staining of HL60 cells with the PE-1 chimera. The P-selectin-IgG, E-selectin-IgG, and PE-1 chimeras were tested for binding to HL60 cells by flow cytometry as in Fig. 1. The percentage of cells staining positively (based on staining with the secondary antibody alone) with each chimera is indicated.

HL60 cells (Fig. 5D). Interestingly, the binding pattern seen with cells closely mimics that seen with the immobilized glycolipid 2'3 sLe<sup>X</sup>. K8A and K84A both bound to HL60 cells, Y48F and K113A did not, and K111A bound HL60 cells only partially. Similar reactivities were seen when neutrophils were stained (data not shown). So, mutation of residues within this pocket of P-selectin also affected binding to its cognate ligand on cells. Furthermore, comparison of the reactivity of this panel of mutants with purified glycolipids provided some potential insights into the nature of the carbohydrate seen by P-selectin (see Discussion).

In E-selectin the arginine at position 97 was also important for sugar recognition. Mutation of this residue to alanine completely abolished E-selectin/2'3 sLe<sup>X</sup> binding (10). The residue at position 97 in P-selectin is a serine and the above results indicated that P-selectin appears to use the same region as E-selectin in binding to its ligand. Therefore, we tested if this difference in residues at position 97 could account for the differences in ligand binding by E- and P-selectin. Examination of the three-dimensional models of the E- and P-selectin lectin domains (Fig. 4) reveals that amino acid 97 falls within a loop formed by residues 94-100, which is an insertion in selectins relative to the mannose binding protein. The sequence of these two selectins is quite different through this stretch -YIKREKDV for E-selectin vs. YIKSP-SAP for P-selectin - so these loops would be expected to have different conformations. To test the importance of the residue at position 97 in conferring specificity to selectins, we made a P-selectin-IgG mutant with the 94-100 loop replaced with the corresponding residues from E-selectin: S97R, P98E, S99K, A100D, and P101V. We then tested this mutant (abbreviated REKDV) for binding to antibodies, glycolipids, and cells. Binding of the P-selectin-IgG REKDV mutant to each of the three anti-P-selectin mAbs (AK-6, AC 1.2, and CRC 81) was  $\sim$ 70% of control P-selectin-IgG binding. This would seem to indicate that although folding of this mutant



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P-selectin	1	MSVNTSTEAMTYDEASANCQDORYTHLVAIOMKEEIEYLN
P-selectin	1	WTYHYSSTKAYSWNISRKYCONRYTDLVAIOMKHEIDYLN
E-selectin	61	SYSPSYWIGIRKVNIVWVWVGTOKPLTEAKNWAPG
E-selectin	61	VUWVSSYYWIGIRKVNIVWVWVGTOKPLTEAKNWAPG
P-selectin	61	PNNRDKDEDCEVIIYIKREKDVGMWNDERCSKKKLALC
P-selectin	61	PNNKRNNEDCCEVIIYIKSPSAPGKWNDEHCKKKHALC

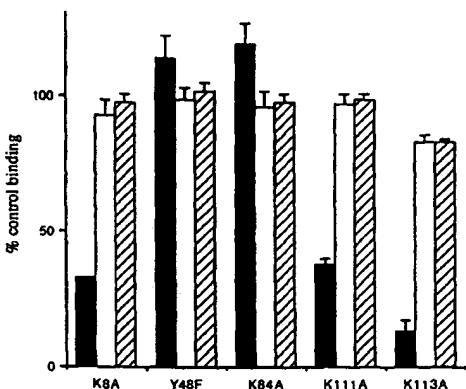
(left) E8-dark green, R84-dark brown, R97-red, and top-to-bottom Y48-K113-K111-yellow. The purple loop (residues 43-48) and the dark blue loop (residues 94-100) in each model denote two insertions near the proposed carbohydrate binding sites that are not found in the mannose binding protein. In each model the single bound calcium is depicted as a light blue ball. (Bottom) Sequence alignment of the human E- and P-selectin lectin domains.

is largely correct, some subtle structural perturbations may be present. Accordingly, this mutant did not bind any of the purified glycolipids in the solid phase ELISA (data not shown). However, the REKDV mutant did stain HL60 cells in the FACS assay, although its binding was significantly less than that seen with control P-selectin-IgG (70% cells positive, MFI 290 for REKDV mutant vs. 97% cells positive, MFI 416 for control P-selectin-IgG). Thus, transferring this loop (containing residue 97) from E- to P-selectin did not completely disrupt the ability of the resultant P-selectin mutant to recognize its cellular ligand. This may indicate that the binding of P-selectin-IgG to its cellular ligand is of higher affinity than binding of P-selectin-IgG to the immobilized glycolipids. Also, these results would seem to imply that at least some of the differences in binding between E- and P-selectin must be due to differences outside of this region (see Discussion).

**Figure 4.** A model of the lectin domain of P-selectin. (Top) Shown is a ribbon model of the P-selectin lectin domain (right) alongside a ribbon model of the E-selectin lectin domain (10) (left). Each model is similarly derived from the published coordinates of the related type C lectin, the mannose binding protein (47) as described (10 and Materials and Methods). Residues shown are colored as follows: for P-selectin (right) K8-light green, K84-light brown, S97-white, and top-to-bottom Y48-K113-K111-yellow, for E-selectin

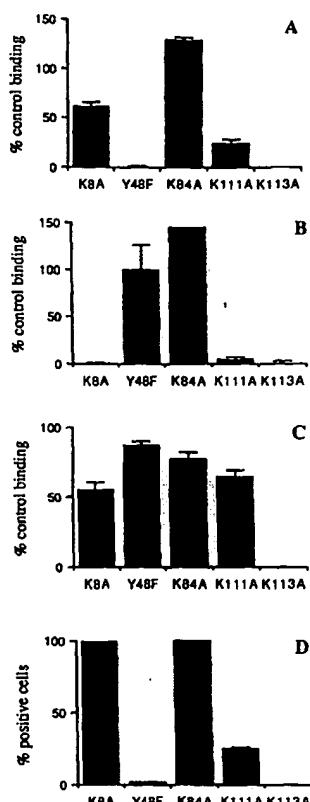
Discussion

Research on selectin-carbohydrate interactions continues to be hampered by a lack of detailed understanding of the sugar structures seen by each adhesion molecule. However, results from a number of approaches, including direct-binding studies, soluble carbohydrate inhibition studies, and structural and conformational analyses of purified potential ligands, have indicated commonalities in selectin recognition. Many of these findings have centered around the sLe<sup>x</sup> core structure. However, many of these proposed similarities may be artifacts of forced binding under experimentally manipulated circumstances (see reference 44 for discussion). In vitro assays with solid phase carbohydrate ligands and transfected, over-expressed selectins can be misleading due to the unnaturally high densities of both receptors and ligands (44). Furthermore, unrelated sugars can inhibit the



**Figure 5.** Reactivity of anti-P-selectin mAbs with mutant chimeras. P-selectin-IgG chimeras with the substitutions indicated were tested for capture by the mAbs AK-6 (■), AC 1.2 (□), and CRC 81 (▨) as in Table I. Results shown represent the mean  $\pm$  SD of duplicate determinations and are expressed as percentage of control P-selectin-IgG binding.

same lectin interaction due to structural mimicry (44). The flow cytometric assay used here to measure P-selectin's interactions with its cellular ligand should avoid most of these limitations while still being sensitive and convenient. The experiments presented here indicate that the measured binding observed using the P-selectin-IgG chimera to stain cells accurately represents the interaction of P-selectin with its ligand. Studies to date have shown that P-selectin binds a single, possibly unique, major glycoprotein of 120 kD (32). The same glycoprotein has been isolated from both neutrophils and HL60 cells (32) and the number of such binding sites for P-selectin is estimated at 10,000–20,000 per cell (31, 32, 37). sLe<sup>x</sup> may form some component of this glycoprotein ligand, and sLe<sup>x</sup> is sufficient to confer some P-selectin binding. However, sLe<sup>x</sup> is not sufficient to confer the saturable, high-affinity binding characteristic of P-selectin adhesion (31). Therefore, the P-selectin ligand must have structural features in addition to sLe<sup>x</sup> that confer specificity and affinity (32, 49). The protein portion of the P-selectin ligand may contribute to this specificity and affinity by: (a) presenting the sugar in the correct configuration; (b) presenting multivalent sugars to enhance binding avidity; and/or (c) participating in a protein–protein contact with P-selectin (32). In fact, a role for presentation of polyvalent ligands to L-selectin by the GlyCAM 1 ligand has already been proposed (27). In the assay described here, P-selectin-IgG binding was ablated by protease treatment of cells, consistent with a requirement for this glycoprotein (24). As noted above, sialic acid is crucial to P-selectin binding and sialidase treatment also abolished binding. Most importantly, removal of calcium chelation by EGTA also led to a loss of binding, a result that is a signature of the interactions mediated by all C-type lectins (47). A surprising finding was that the E-selectin-IgG chimera did not bind HL60 cells or neutrophils in this fluid phase staining assay. This is despite the fact that the E-selectin carbohydrate ligand, sLe<sup>x</sup>, is clearly expressed by these cells (33, 45). Furthermore, we (10) and others (41) have found that E-selectin-IgG is capable of binding HL60 cells and neutrophils when the chimera is



**Figure 6.** Binding of P-selectin-IgG mutants to immobilized glycolipids and cells. P-selectin-IgG chimeras with the substitutions indicated were tested for binding to immobilized 2'3 sLe<sup>x</sup> glycolipid (A), 2'6 sLe<sup>x</sup> glycolipid (B), or sulfatides (C) as in Fig. 2, and for staining of HL60 cells (D) as in Fig. 1.

presented on a solid substrate, suggesting that the lack of binding in the fluid phase may be due to lower affinity of E-selectin for its cognate cell surface ligand. Thus, E- and P-selectin are clearly distinct in binding to cells both as soluble Ig chimeras, as well as when they are expressed on endothelial/platelet cell surfaces.

At least part of this difference between E- and P-selectin must be due to differences in their lectin domains. Transferring the P-selectin lectin domain onto the E-selectin-IgG construct resulted in a molecule (PE-1) which stained cells, albeit at a lower intensity than P-selectin-IgG. Carbohydrate reactivity was completely transferred with the relevant lectin domain. Thus, PE-1 reacted with the purified glycolipids in a manner that was indistinguishable from P-selectin-IgG and quite distinct from E-selectin-IgG. Therefore, the lectin domain of each selectin appears sufficient for determining the differences in reactivities with these relatively small sugars. This result is consistent with a study by Kansas et al. (20) in which domains of L- and P-selectin were exchanged to show that PPME and fucoidin binding, both L-selectin-specific carbohydrate ligands, as well as the epitope defined by blocking mAb LAM1-3, map at least in part to the COOH-terminal 67 amino acid residues of the L-selectin lectin domain. These authors also demonstrated that the CR domains are not important for conferring PPME or fucoidin specificity (20). The EGF and CR domains of selectins have clearly been shown to perform vital structural roles for these receptors (6, 19, 45, 46). Whether these domains in P-selectin also participate in making crucial contacts with its glycoprotein ligand cannot be answered here. However, the results of this study do place limitations on the nature of any such contacts.

First, the P-selectin-IgG chimera used here only contains the lectin, EGF, and CR1 domains of P-selectin (12). Thus, CR2-CR9 must not form necessary contacts for the high affinity binding between P-selectin and its ligand and it is interesting to note that mouse P-selectin lacks the CR2 domain (48). In addition, because the PE-1 chimera did bind cells, any potential protein-protein contact sites may map to the lectin domain of P-selectin. The difference in staining between PE-1 and P-selectin-IgG might reflect subtle conformational effects of the P- or E-selectin EGF domains interacting with the common lectin domain. However, it is important to stress that protein-protein contacts mediated by the EGF or CR1 domains cannot be ruled out.

Two recent studies have identified regions of the P-selectin lectin domain that may be important for cell adhesion (14, 15). Geng and co-workers showed that a mAb capable of inhibiting neutrophil binding to P-selectin mapped to residues 19–34 of this molecule, and that a peptide corresponding to this stretch also inhibited neutrophil binding to P-selectin (14). This group described other peptides from the lectin domain of P-selectin (corresponding to residues 23–30, 54–63, and 70–79) which blocked P-selectin-mediated adhesion (15). In the model of P-selectin, these residues fall on the opposite side of the lectin domain from the site that we have identified as important for selectin-carbohydrate binding and cell adhesion (see Fig. 4 and reference 10). The residues characterized by Geng and coworkers may represent a second site in P-selectin which may bind the carbohydrate and/or protein component of its ligand. In light of this, it is important to remember that the results with the REKD mutant indicated that not all of the differences in specificity between E- and P-selectin can be explained by the region identified in this study. Therefore, the possible co-operation of this site with those described by Geng et al. (14, 15) in conferring P-selectin binding specificity warrants exploration.

The results presented here establish that the site previously identified as crucial for E-selectin binding to 2'3 sLe<sup>x</sup> is also crucial to P-selectin binding to this ligand. Mutations in two of the conserved residues within this site, Y48 and K113, completely abolished 2'3 sLe<sup>x</sup> binding and cell adhesion by both E- and P-selectin. The anti-P-selectin blocking mAb AK-6 mapped to this same site, as did all of the anti-E-selectin blocking mAbs (10). Furthermore, Mel-14, a mAb that blocks L-selectin-mediated adhesion in vitro and in vivo, maps to this region (6, 10). The fact that adhesion blocking mAbs to all three selectins bind to residues within this site emphasizes its importance to the adhesive functions of these proteins.

By comparing the binding of the panel of P-selectin mutants to 2'3 sLe<sup>x</sup>, 2'6 sLe<sup>x</sup>, and sulfatides with their ability to bind cells, some insight as to the nature of the carbohydrate component of the P-selectin ligand can be gained. As noted above, one study has shown that E- and P-selectin have related but distinct carbohydrate specificities (24). For example, these authors found that the interaction of E-selectin with the sLe<sup>x</sup> component of the P-selectin ligand precludes P-selectin binding (24). Using a 2'6 sialyl-specific lectin to block P-selectin binding, they also proposed that the P-selectin ligand may contain a bidentate carbohydrate structure with one arm containing 2'3 sLe<sup>x</sup> and the other a terminal sialyl-2'6 beta Gal (13, 24). However, our results with

the P-selectin mutants would seem to question a role for 2'6-linked sialic acid in cell adhesion. The mutant K8A did not bind the 2'6 form of sLe<sup>x</sup> at all, but still bound the P-selectin ligand on cells. Furthermore, the Y48F mutant did not bind cells at all, but still bound 2'6 sLe<sup>x</sup>. Consequently, 2'6 sLe<sup>x</sup> binding did not correlate with ligand binding. However, the binding to 2'6 sLe<sup>x</sup> in the solid phase assays used here is weak compared to 2'3 sLe<sup>x</sup> and sulfatide binding, so caution is warranted in interpreting these results. Thus, it is conceivable that presentation of the 2'6 sialylated carbohydrate to P-selectin provides a critical parameter of ligand recognition that is not replicated in our solid phase assay (27).

A second binding activity of P-selectin whose biological relevance has been recently questioned is its interaction with sulfatides. Sulfatide binding by P-selectin is probably not relevant in vivo due to the observation that this interaction would be protease resistant, and that cells expressing sulfatides (erythrocytes and platelets) do not necessarily bind P-selectin (32). Also, sulfatide binding by the P-selectin mutants studied here did not correlate with cell binding. For example, mutant Y48F bound sulfatides well but did not adhere to cells at all. Cell binding was only correlated with binding to 2'3 sLe<sup>x</sup>. Each mutant which bound 2'3 sLe<sup>x</sup> bound cells (K8A and K84A), while those which did not bind 2'3 sLe<sup>x</sup> (Y48F and K113A) did not bind cells, and one mutant (K111A) showed partial binding to both 2'3 sLe<sup>x</sup> and cells. This is interesting given a recent study demonstrating that expression of 2'3 sLe<sup>x</sup> correlated with a cell's ability to bind activated platelets via P-selectin (9), and it is consistent with the mAb and carbohydrate blocking studies of Polley et al. (34).

Although one cannot rule out the involvement of 2'6-linked sialic acid or sulfatides in P-selectin's interactions with its ligand, the data presented here clearly questions the role they may play. Specificity of binding between E- and P-selectin may derive from the manner in which 2'3 sLe<sup>x</sup> is presented (i.e., glycolipid vs. glycoprotein). However, it must be allowed that 2'3 sLe<sup>x</sup> may not be the naturally occurring carbohydrate ligand recognized by either selectin, and that these differences in selectin binding could be accounted for by subtle changes in the saccharide itself (44). Sulfatides, as well as the sulfated glycans heparin, fucoidin, and dextran sulfate, may inhibit P-selectin function by mimicking its ligand (32). sLe<sup>x</sup>, sulfated glycans, and sulfatides all have a negative charge which may play a role in the interaction of P-selectin with its ligand (9, 10) and these sugars may inhibit selectin-mediated adhesion by binding to a common site (for example, at K113) which is important for P-selectin-ligand interactions.

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